Normal Segregation of a Foreign-Species Chromosome During *Drosophila* Female Meiosis Despite Extensive Heterochromatin Divergence

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ABSTRACT The abundance and composition of heterochromatin changes rapidly between species and contributes to hybrid incompatibility and reproductive isolation. Heterochromatin differences may also destabilize chromosome segregation and cause meiotic drive, the non-Mendelian segregation of homologous chromosomes. Here we use a range of genetic and cytological assays to examine the meiotic properties of a *Drosophila simulans* chromosome *4* (*sim-IV*) introgressed into *D. melanogaster*. These two species differ by ~12–13% at synonymous sites and several genes essential for chromosome segregation have experienced recurrent adaptive evolution since their divergence. Furthermore, their chromosome *4*s are visibly different due to heterochromatin divergence, including in the AATAT pericentromeric satellite DNA. We find a visible imbalance in the positioning of the two chromosome *4*s in *sim-IV/mel-IV* heterozygote and also replicate this finding with a *D. melanogaster 4* containing a heterochromatic deletion. These results demonstrate that heterochromatin abundance can have a visible effect on chromosome positioning during meiosis. Despite this effect, however, we find that *sim-IV* segregates normally in both diplo and triplo *4 D. melanogaster* females and does not experience elevated nondisjunction. We conclude that segregation abnormalities and a high level of meiotic drive are not inevitable byproducts of extensive heterochromatin divergence. Animal chromosomes typically contain large amounts of noncoding repetitive DNA that nevertheless varies widely between species. This variation may potentially induce non-Mendelian transmission of chromosomes. We have examined the meiotic properties and transmission of a highly diverged chromosome *4* from a foreign species within the fruitfly *Drosophila melanogaster*. This chromosome has substantially less of a simple sequence repeat than does *D. melanogaster 4*, and we find that this difference results in altered positioning when chromosomes align during meiosis. Yet this foreign chromosome segregates at normal frequencies, demonstrating that chromosome segregation can be robust to major differences in repetitive DNA abundance.

HETEROCHROMATIC repeats at and near telomeres and centromeres turn over rapidly at short evolutionary time scales (Charlesworth *et al.* 1994). A subset of genes involved in meiosis, chromosome and chromatin function, and transposable element defense also show high rates of divergence between sibling species, often with accompanying signatures of adaptive evolution (Malik and Henikoff 2001; Begun *et al.* 2007; Larracuente *et al.* 2008; Anderson *et al.* 2009; Obbard *et al.* 2009; Raffa *et al.* 2011; Langley *et al.* 2012). These patterns suggest that organisms need to mount a continual adaptive response to suppress deleterious consequences caused by heterochromatic repetitive DNAs. Satellite DNAs and transposable elements, the major components of heterochromatin, can increase their copy numbers by unequal crossing over and transposition. These expansions can reduce fitness by increasing genome size and rates of ectopic recombination.

Repetitive DNA evolution can be particularly rapid if it selfishly biases its transmission through meiosis (true meiotic drive) or gametogenesis (gametic drive; we refer to both phenomena collectively as segregation distortion). Meiotic drive is an especially strong driver of chromosomal evolution that takes advantage of asymmetric meioses (that is, females in *Drosophila* and mammals) where only one meiotic product

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Table 1 Test of segregation, chromosome loss, and NDJ

		Regular progeny			Exceptional progeny	
Chr. *4* tested	F_1 sex	No. inheriting P[y$^+$]	No. inheriting tested chromosome	Segregation ratio[a]	No. *4* NDJ	*4* NDJ %[b]
w$^+$-IV	Female	1249	1194	0.489	1	
	Male	1022	1095	0.517	1	
	Both	2271	2289	0.502 N.S.	2	0.044
sim-IV	Female	1276	1147	0.473	0	
	Male	1031	963	0.483	1	
	Both	2307	2110	0.478**	1	0.023

y w; *w$^+$-IV* females were crossed to *w/Y*; *sim-IV/ci^D* males. *y w/Y*; *w$^+$-IV/sim-IV* sons were then crossed to *y w*; *y$^+$-IV* females. *y w*; *y$^+$-IV/w$^+$-IV* and *y w y$^+$-IV/sim-IV* daughters were collected and separately crossed to *y^1 pn^1/Y*; *C(4)RM*, *ci^1 ey^R/O* males at 27°.

[a] Defined as the ratio of those inheriting the tested chromosome/total progeny. As each class has a 50% chance of survival due to sperm genotype (Figure 5), significance was tested by comparison to simulation of equal segregation followed by 50% survival with 1,000,000 replicates. N.S., not significant ($P > 0.5$); **$P < 0.002$.

[b] Calculated as the number of observed exceptional progeny/total progeny (excluding *minutes*; see Figure 5 and *Materials and Methods*). The NDJ rates for the two genotypes were not significantly different ($P = 1$, Fisher's exact test).

becomes the egg pronucleus (Pardo-Manuel De Villena and Sapienza 2001; Fabritius *et al.* 2011). The selfish elements that cause meiotic drive likely result from variation in heterochromatic repeat sequences (Buckler *et al.* 1999; Fishman and Saunders 2008). Adaptive divergence of centromeric and telomeric proteins may reflect a host response to suppress meiotic drive, as meiotic drivers can have pleiotropic deleterious consequences on host fitness (Zwick *et al.* 1999; Henikoff *et al.* 2001).

There are hints that segregation distorters may be prevalent in natural populations (Jaenike 2001; Reed *et al.* 2005; Bastide *et al.* 2013), but few specific loci have been identified. Hybrid backgrounds may reveal these loci, if suppressors fail to function or are separated from their targets by segregation (Mercot *et al.* 1995). Here we take advantage of a rare opportunity to examine meiotic transmission of an entire foreign chromosome, which is *D. simulans* chromosome *4* (*sim-IV*) in a heterospecific *D. melanogaster* background. *D. melanogaster* and *D. simulans* are sibling species that can be intercrossed but contain substantial divergence. Alignable synonymous nucleotide sites are ~12–13% diverged (Begun *et al.* 2007), and the species are strikingly different in repetitive DNA content and heterochromatin, with *D. simulans* having substantially fewer transposable elements and less satellite DNA (Lohe and Roberts 1988; Bosco *et al.* 2007; Lerat *et al.* 2011). They also have experienced adaptive evolution in genes that are essential for chromosome segregation (Malik and Henikoff 2001; Anderson *et al.* 2009).

Chromosome *4* has a number of advantages for this study. (1) *sim-IV* is viable when introgressed into *D. melanogaster* due to its small size, the only incompatible phenotype being homozygous male sterility (Muller and Pontecorvo 1942). (2) Chromosome *4* is triplo-viable, which allows for novel chromosome segregation assays (Sturtevant 1934). (3) Chromosome *4* contains an interesting mix of heterochromatic and euchromatic properties (Riddle *et al.* 2009). It has a high proportion of repetitive DNA but a normal abundance of protein coding genes. It is therefore not a gene-poor *B* or *Y* chromosome. (4) Chromosome *4* is achiasmatic and segregates in the absence of crossing over. Therefore all divergence on *4* remains linked to the centromere and can potentially impact meiotic segregation. (5) Chromosome *4* segregation nevertheless typically utilizes homology to achieve pairing during meiosis, while also being able to segregate under an alternative homology-independent pathway when homology is absent (Hawley *et al.* 1992). In short, we propose that we are testing for faithful segregation among the most diverged chromosomes possible in an animal model.

One recent advance in understanding the segregation of nonexchange chromosomes, such as the small *4* chromosomes of *Drosophila*, is the identification of tethers connecting spatially separated chromosomes during prometaphase of meiosis I in females. These tethers appear to be built from pericentromeric heterochromatin and are proposed to establish tension between chromosomes not held together by chiasmata, thus allowing homologous coorientation to be established (Hughes *et al.* 2009, 2011). Similar tethers have been inferred by micromanipulation experiments in grasshopper spermatocytes (LaFountain *et al.* 2002) and by PICH localization to DNA threads connecting mitotic sister kinetochores in mammalian cultured cells (Baumann *et al.* 2007). While the exact mechanisms of establishing and resolving these tethers are unknown, they are a strong candidate for establishing nonexchange chromosome segregation, as heterochromatic homology is sufficient for coorientation (Hawley *et al.* 1992). Heterochromatin divergence between species can cause mitotic segregation failure in interspecific hybrids (Ferree and Barbash 2009). Here we address whether a foreign-species chromosome with extensive divergence affects the formation of heterochromatic threads and can segregate properly during female meiosis.

Materials and Methods

Drosophila stocks and nomenclature

We refer to generic fourth chromosomes as *4*, and specific fourth chromosomes as *IV*. Therefore, the unmarked introgressed *D. simulans 4th* chromosome used in this study is referred to as *sim-IV*. An exception is the *D. melanogaster* chromosome *4* containing the visible eye marker *sv$^{spa\text{-}pol}$*, which we refer to simply as *pol*. The *4* wild-type lines used in triplo-*4*

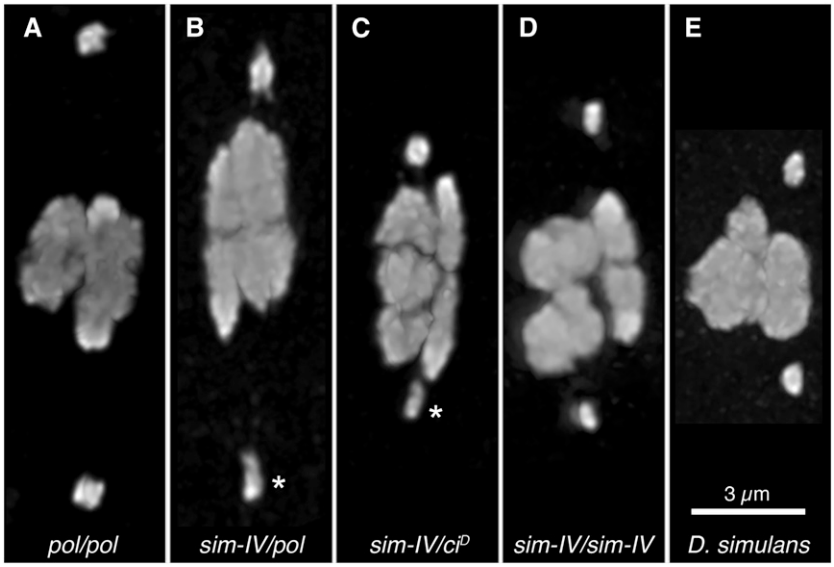

Figure 1 Asymmetry in *sim-IV* heterozygotes. *pol* and ci^D are visible markers on different *D. melanogaster* chromosome *4*s. Representative oocytes from 42- to 48-hr-old mated females from the DAPI-only preps used for *4-4* distance measurement, scaled to the same size. The differences in the brightness of the *4*s are not as clear in these projected images as in the ocular, so the background-subtracted intensity of each *4* was determined, and the brightness ratio (dimmer *4*/brighter *4*) calculated, for 10 oocytes per genotype, with the mean (and range) reported. (A) Homozygous control *pol/pol* oocyte. Mean brightness ratio: 0.87 (0.77–0.98). (B) Heterozygous *sim-IV/pol* oocyte made from outcrossing the introgression stock. The dimmer *sim-IV* chromosome is indicated (asterisk). Mean brightness ratio: 0.63 (0.40–0.76). (C) Heterozygous *sim-IV/ci*^{D} oocyte from the introgression stock. The dimmer *sim-IV* chromosome is indicated (asterisk). Mean brightness ratio: 0.66 (0.57–0.89). (D) Homozygous *sim-IV/sim-IV* oocyte from the introgression stock. The *4*s are dimmer but not asymmetric. Mean brightness ratio: 0.88 (0.73–0.96). (E) Pure-strain *D. simulans* oocyte. The *4*s are also dimmer but not asymmetric. Mean brightness ratio: 0.94 (0.78–0.99).

segregation assay were obtained from Stuart MacDonald and are described elsewhere (King *et al.* 2012). We created a *D. melanogaster y w sim-IV/ci*^{D} stock derived from the *sim-IV* introgression obtained from J. P. Masly (Masly *et al.* 2006). All other stocks were from the Hawley lab or obtained from the Bloomington *Drosophila* Stock Center. We used a w^+-marked chromosome *4* (y^1 w^{1118}; *PBac{*w^{+mC} = *5HPw*$^+$*}CG33978*^{A437}), abbreviated as w^+*-IV* as a control chromosome in crosses in Table 1 to measure *sim-IV* segregation and production of nullo maternal gametes. A y^+-marked chromosome *4* (y^1 w^{1118}; *PBac{*y^+*-attP-9A}VK00024*), abbreviated as y^+*-IV*, was used as the opposing chromosome to follow segregation of the *sim-IV* or control chromosome.

Drosophila crosses

In the *C(4)RM*, ci^1 ey^R stock used in Table 1, the penetrance of the *ey* phenotype was variable. Among the thousands of progeny, a small number of various developmental defects were observed. Therefore flies were scored as being *ci ey* only if both wings displayed the ci^1 phenotype and at least one eye displayed a small or misshapen eye characteristic of the ey^R phenotype. In the experimental cross *ci ey* females will be *y* w^+, and *ci ey* males will be *y w*. Regular progeny with these phenotypes are thus potentially overlapping with *C(4)/O* if the regular progeny have morphological defects affecting the wings and eyes. Between 2 and 11 flies with morphological defects were found for each sex and genotype in the Table 1 crosses and were predominantly cases where one eye was missing and wings were wild type or where both eyes were wild type and one wing had a defective longitudinal vein 4 or 5. In the control cross *ci ey* females will be *y* w^+, and *ci ey* males will be *y w*. No regular *y w* males will be produced but regular *y* w^+ daughters are again potentially overlapping with *C(4)/O*. We also found the minute phenotype associated with haplo-*4* challenging to score but classified between 2 and 17 flies of each sex and genotype as *minute* in Table 1.

To measure nondisjunction (NDJ) in the *y w*; *sim-IV/sim-IV*, *y w*; *sim-IV/pol* and *y w*; *sim-IV/ci*^{D} genotypes, single virgin females were mated to multiple *C(1;Y)*, *v f B/O*; *C(4)RM*, *ci ey*^{R}*/O* males in vials, allowed to lay eggs for 5 days, and adults removed. *X* chromosome NDJ could be seen by following *y* (normal progeny were y^+ females and y^- males, while progeny of diplo-*X* or nullo-*X* eggs were y^- females and y^+ males, respectively). Progeny of nullo-*4* eggs could be identified as being both *ci* and *ey* (normal progeny in the *sim-IV/ci*^{D} cross could be *ci* alone), but because the *sim-IV* chromosome is wild type for all chromosome *4* markers, diplo-*4* progeny of mothers carrying *sim-IV* could not be distinguished from normal progeny.

To produce *y w*; *sim-IV/pol* females, we crossed *y w*; *sim-IV* homozygous females from the introgression stock to males from a *y w/y*$^+$*Y*; *pol* laboratory stock. Then *y w/y*$^+$*Y*; *pol/sim-IV* heterozgous males were collected and backcrossed to *y w*; *pol* virgin females to produce *y w*; *pol/sim-IV* females.

To produce *FM7*, *y w B/y w*; *pol/sim-IV* and *FM7*, *y w B/y w*; *sim-IV/sim-IV* females, *y w/y*$^+$*Y*; *sim-IV/pol* males from above were crossed to *FM7*, *y w B*; *pol* females, and *FM7*, *y w B/y*$^+$*Y*; *sim-IV/pol* males and *FM7*, *y w B/y w*; *sim-IV/pol* virgin females were collected. These were sib-mated, which produced *FM7*, *y w B/y w* females that were phenotypically *pol*$^+$. These females could be either *pol/sim-IV* or *sim-IV/sim-IV*, which were expected in a 2:1 ratio. These females were mated singly in vials to *C(1;Y)/O*; *C(4)/O* tester males to test *X* and *4* NDJ as above. The maternal *4* genotype was inferred to be *sim-IV/pol* if any *pol minute* progeny were produced in a vial. Vials that did not produce any *pol minute* progeny were also testcrossed by mating multiple F_2 females to *y w/y*$^+$*Y*; *pol* males and looking for any *pol* progeny; all tested vials were confirmed

to lack *pol*, meaning the experimental female in that vial must have been *sim-IV/sim-IV*. Count data for each vial were then combined by maternal *4* genotype.

To produce *y w/y w nod^{a}*; *pol* and *y w/y w nod^{a}*; *sim-IV/pol* progeny, *y w nod^{a}/y^{+}Y*; *pol* males (from a stock with the *X* balanced over *C(1)DX* females) were crossed to *FM7*, *y w B/y w*; *pol/sim-IV* virgin females from above, and virgin females of both genotypes were collected and mated singly in vials to *C(1;Y)/O*; *C(4)/O* tester males as above.

To produce triplo-*4* females, we used a mutation in *nod* to increase the rate of nondisjunction. The *w^{+}-IV* chromosome was crossed into a *FM7a*, *nod* background to generate the stock *C(1)DX*, *y^{1} w^{1} f^{1}/FM7a*, *nod^{4}/ /Dp(1;Y)y^{+}*; *PBac {w^{+mC} = 5HPw^{+}}CG33978^{A437}*. We abbreviate the males from this stock as *FM7a*, *nod^{4}/Y*; *w^{+}-IV*. To generate triplo-*4* females, we first crossed *y w*; *y^{+}-IV* females to *FM7a*, *nod^{4}/Y*; *w^{+}-IV* males. F_1 virgin daughters of genotype *y w/FM7a*, *nod^{4}/Y*; *y^{+}-IV/w^{+}-IV* were then mated to males of genotype *y w/Y* containing different chromosome *4* genotypes. Males containing wild-type chromosome *4*s were generated by crossing *y w*; *sim-IV/ci^{D}* females to wild-type males and selecting *y w/Y*; *+/ci^{D}* sons. Rare *y w/y w* daughters inheriting both maternal chromosome *4*s and a paternal chromosome *4* were identified by their y^{+} w^{+} phenotype; where appropriate non-ci^{D} females were selected in order to obtain the desired paternally inherited wild-type chromosome *4*. Triplo-*4* females were then mated singly to 2 *y w/Y* males at 25°.

Probability analyses were done in R (cran.r-project.org). To test significance for random segregation with 50% survival in Table 1, a binomial number N_j was generated with a mean of 0.5 and an *N* of twice the experimental result. The surviving segregation proportion was then simulated as p_j = binomial (0.5, N_j)/N_j. This was repeated 1,000,000 times to generate a distribution, with significance determined as the two-tailed likelihood of obtaining the observed result due to chance.

4-4 distance preps

Bottles were cleared of adults and virgin females of the desired genotypes were collected 6 hr later. Females were aged in yeasted vials with sibling males for 42 hr after collection, and so were 42–48 hr posteclosion at the point of dissection. To standardize prep conditions, a timer was started as the vial was anesthetized with CO_2, followed by hand dissection of ovaries as quickly as possible in room temperature 1× Robb's media + 1% BSA (Matthies *et al.* 2000), transferring ovaries to a second well of media after extraction. After 10 females were dissected, the ovaries were left to incubate in Robb's until the timer reached 7 min, when buffer plus ovaries were pipetted into a 1.5-ml Eppendorf tube and allowed to settle. At 8 min, the Robb's was aspirated, and 1.3 ml of room temperature fixative [a 1:1 mix of 16% EM grade paraformaldehyde (Ted Pella) with William's Hypotonic Oocyte Preservation and Stabilization Solution (Gillies *et al.* 2013), combined just before use] was applied. After fixation at room temperature for 5 min, oocytes were washed briefly in PBST (PBS + 0.1% Triton X-100), ovarioles were separated by rapid pipetting with a p1000 pipette, washed three times in PBST for 15 min each, stained in PBST plus 1× DAPI for 6 min, washed in PBST (three times quickly followed by two times for 15 min) then mounted on slides in SlowFade Gold (Invitrogen).

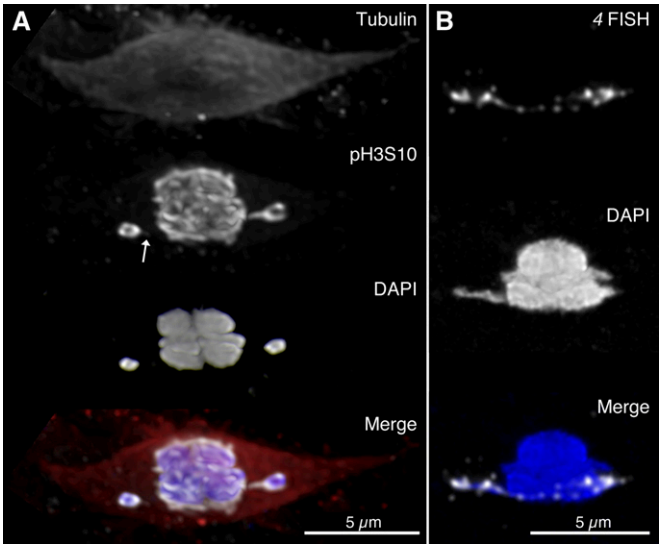

Figure 2 Heterochromatin threads in *D. simulans*. (A) Fixed oocyte from a 2-day-old mated *D. simulans* female, visualized by immunofluorescence with anti-tubulin (red), anti-pH3S10 (white), and DAPI (blue) staining. Threads are detectable by anti-pH3S10 the right chromosome has a clear and complete thread while a very dim spur can be seen on the left chromosome (arrow). (B) Fixed oocyte from a 3-day-old mated *D. simulans* female, visualized by heterochromatin FISH (white) against the AATAT repeat primarily found on chromosome *4*. A complete thread can be detected running between the *4* chromosomes.

Fluorescent in-situ hybridization preps

Females were aged for 2 or 3 days posteclosion in yeasted vials with males. A timer was started as females were anesthetized with CO_2, transferred to a CO_2 plate for 1 min, then the gas was turned off, flies were covered with a Petri dish lid, and allowed to rest on the plate. At 6 min, the CO_2 was turned back on, and ovaries were dissected as quickly as possible in Robb's (above). Once all ovaries were dissected, they were left to incubate in Robb's until 15 min from the start of the procedure, when they were transferred to an Eppendorf tube. Oocytes were allowed to settle for 1 min, the Robb's was aspirated, and 1.3 ml of prewarmed 39° fixative (above) was applied. Oocytes were fixed for 4 min at 39°, washed briefly in 2× SSCT (saline sodium citrate + 0.1% Tween 20), and ovarioles separated by pipetting. Oocytes were washed in 2× SSCT three times for 10 min, washed 10 min each in 2× SSCT containing 20, 40, and 50% formamide, then incubated in 2× SSCT + 50% formamide for 2 hr at 37°. As much buffer as possible was aspirated, and 40 μl of hybridization solution (36 μl of 1.1× hybridization solution (1.0 g dextran sulfate, 1.5 ml 20× SSC, 5.0 ml formamide, dilute to 9.0 ml with ddH_2O) plus 4 μl of probe mix) was added. All

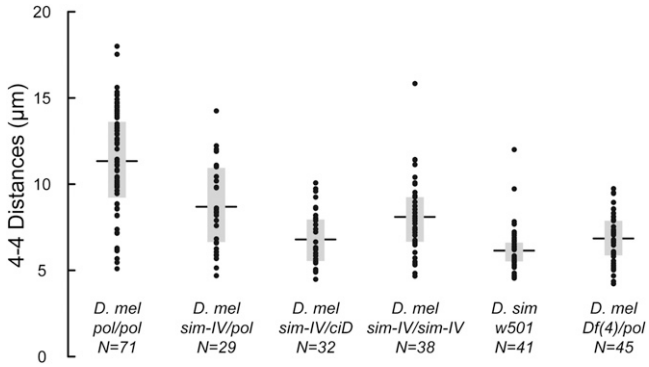

Figure 3 *4-4* distance measurements. *pol* and ci^D are visible markers on different *D. melanogaster* chromosome *4*s. (A) The mean distances for each genotype (horizontal lines) and the inner quartile ranges (boxes) are indicated, along with the number of measurements. The first four sets are for *D. melanogaster*, including the *pol/pol* control, the outcrossed *sim-IV/pol* heterozygote, the introgressed *sim-IV/ci*^{D} heterozygote, and the introgressed *sim-IV/sim-IV* homozygote, while the fifth set is for pure-strain *D. simulans* females. The sixth set is *D. melanogaster* females heterozygous for the deletion *Df(4)m101-62f/pol* (see Figure 4).

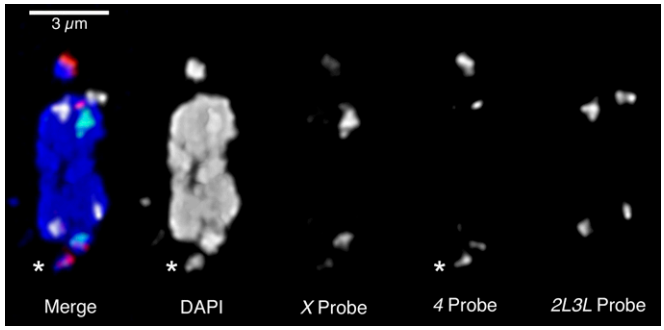

Figure 4 Asymmetry in *Df(4)m101-62f* heterozygotes. A fixed oocyte from a mated 2-day-old heterozygous *Df(4)m101-62f/pol* female is shown, with FISH staining of the 359-bp satellite (*X* probe, green), the AATAT repeat (*4* probe, red), and the AATAACATAG repeat (*2L3L* probe, white) along with DAPI (blue). The *Df(4)* chromosome (asterisk) stains less brightly with both DAPI and the *4* probe, consistent with the deletion of some AATAT heterochromatin from this chromosome.

probes were synthesized with fluorophores by www.idtdna.com and diluted to 200 ng/μl in ddH_2O. Probe mixes were prepared by combining 2 μl of each probe to be used, then diluting to a total volume of 96 μl in ddH_2O, then storing at −20°. For each prep, 4 μl of probe mix was used, resulting in 16.7 ng of each probe in each prep. Probes used were *2L-3L* $(AATAACATAG)_3$ and *4* $(AATAT)_6$ (Dernburg 2000) and *X* (TTT-TCC-AAA-TTT-CGG-TCA-TCA-AAT-AAT-CAT) (Ferree and Barbash 2009).

After the hybridization solution was added, DNA was denatured at 92° followed by overnight hybridization at 32°. Oocytes were washed twice for 15 min in 2× SSCT + 50% formamide at 32°, for 10 min each in 2× SSCT containing 40, 20, and 0% formamide, then stained in 2× SSCT + 1× DAPI for 10 min. Oocytes were washed in 2× SSCT (two times briefly and two times for 10 min), then mounted in SlowFade Gold.

Immunofluorescent preps

Two-day mated females were dissected as per fluorescent *in-situ* hybridization (FISH) preps (1 min CO_2, 5 min rest, quickly dissected then incubated for up to 10 min in Robb's), followed by fixation at room temperature in 1.3 ml fixative. Oocytes were then washed briefly in PBST, ovarioles separated by pipetting, and washed three times for 10 min in PBST. Oocytes were dechorionated by rolling between frosted glass slides, washed three times briefly in PBST, transferred to an 0.5 ml Eppendorf tube, and blocked for 1 hr in PBST-NGS (Matthies *et al.* 2000). Fresh PBST-NGS with primary antibodies (Serotec MCA786 rat antitubulin at 1:250 and Millipore rabbit antiphosphorylated histone H3 at serine 10 at 1:500) was added and hybridized overnight, followed by washing in PBST (three times briefly and once for 15 min), 1 hr blocking in PBST-NGS, and then either a 4-hr incubation at room temperature or overnight at 4°, in PBST-NGS plus secondary antibodies (goat antirat IgG with Alexa Fluor 647 conjugate and goat antirabbit IgG with Alexa Fluor 568 conjugate, Invitrogen, both at 1:250). A total of 2.5 μl of 200× DAPI was added and incubated for 6 min, followed by PBST washes (three times briefly and twice for 15 min) and mounting in SlowFade Gold.

Imaging and quantification

To ensure oocytes were not missed or double counted, microscope slides were photographed on a dissection microscope and a print of the photo was used as a map to mark oocytes. Oocytes were viewed at low magnification and marked using the LAS AF software (www.leica.com) "mark and find" panel. All confocal images were collected with the ×63 objective on a Leica TCS SPE II confocal microscope using LAS AF, and presented images were deconvolved using Huygens Essential (www.svi.nl).

Estimation of *4-4* distances was done by combining XY distances (determined by the LAS AF line tool in projected stacks) with *Z* distances (determined by multiplying the number of confocal sections between the centers of the *4* light cones by the section thickness in orthogonal projections) using the Pythagorean theorem (distance = $\text{sqrt}(xy^2 + z^2)$) in Excel. Measurement was restricted to oocytes that had at least one *4* out on the spindle. This was determined by whether there was at least a 50% dip in background-subtracted fluorescent intensity, measured on the *4* and the space between the *4* and the adjacent chromosome using the line ROI tool. Oocytes with both *4*s on the same side of the spindle, with additional nonexchange chromosomes, or with chromosomes in the "slippage" configuration (Hughes *et al.* 2011) were counted as having chromosomes out on the spindle, but their *4-4* distances were not included in the analysis. Plots and *t*-tests were then done in R.

To calculate chromosome *4* brightness ratios, figures where both *4* chromosomes were fully separated from other chromosomes were selected, identically sized regions of interest (ROI) were placed over each *4* and on nearby empty space, and the summed pixel intensity for each ROI was

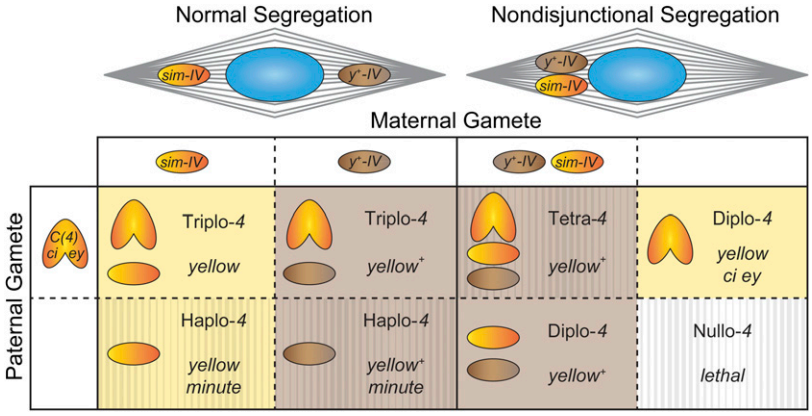

Figure 5 Expected progeny from the cross in Table 1 to measure the *sim-IV* segregation ratio. At top are two spindle diagrams, showing normal segregation (left) and meiosis I nondisjunctional segregation (right). As either spindle pole can form the egg pronucleus, those poles drop down to four types of female gametes in the table. Chromosome loss is also possible but not diagrammed; in that case, nullo-*4* gametes equivalent to the last column will be produced. Females are mated to *compound-4* bearing males of genotype *C(4), ci ey*, who produce either diplo-*4* or nullo-*4* gametes. Progeny will be y^+ if the maternal y^+-*IV* is transmitted, and are otherwise *y* mutant, indicated by the background color. The hatching pattern indicates progeny that are semiviable or lethal. Haplo-*4* leads to *minute* phenotypes with poor viability, while nullo-*4* is always lethal. Tetra-*4* flies from nondisjunctional oocytes are usually lethal, but can survive under some circumstances (Grell 1972). Note that the normal *yellow*$^+$ triplo-*4* progeny are indistinguishable from the nondisjunctional diplo-*4* progeny (as well as any tetra-*4* progeny that survive). Therefore only the *yellow ci ey* class of progeny from NDJ can be observed. A similar situation arises in most of the crosses in Table 2, where *sim-IV/pol* progeny arising from nondisjunction are phenotypically wild type and cannot be distinguished from triplo-*4* regular progeny. In both Table 1 and Table 2, progeny inheriting no maternal *4* are products of either maternal nondisjunction or chromosome loss and are detected by their *ey ci* phenotype. Although only half of the exceptional progeny are therefore detectable, we have calculated *4* NDJ without doubling the number of nullo-*4* progeny observed, as spontaneous *4* NDJ events in wild-type and *nod*$^-$ heterozygous backgrounds yielded 11 nullo events and only 1 diplo event across multiple experimental controls (Zhang and Hawley 1990; Rasooly *et al.* 1991; Gillies *et al.* 2013), suggesting these arise primarily from loss events rather than nondisjunction. Products of meiosis II nondisjunction are not shown, but again only those inheriting no maternal *4* are phenotypically distinguishable.

recorded. The brightness ratio (lower intensity − background)/(higher intensity − background) was calculated for 10 oocytes for each genotype.

Results

Reduced heterochromatin of sim-IV

In examining *sim-IV*, in comparison with pure-strain *D. melanogaster* and *D. simulans* oocytes, we found that *sim-IV* is dimmer than its *D. melanogaster* homolog in DAPI fluorescence. This was readily apparent even in the ocular, and caused an asymmetry between the *4*s in heterozygous females (Figure 1, A–C). This dimness, without asymmetry, was also observed in introgressed *sim-IV* homozygotes (Figure 1D) as well as *D. simulans* females (Figure 1E). This result is not unexpected; the AATAT heterochromatin repeat, which primarily labels the *4* in females (Dernburg 2000), is considerably less abundant in the *D. simulans* genome, comprising only 1.9% of the genome *vs.* 3.1% in *D. melanogaster* (Lohe and Brutlag 1987).

Positioning of sim-IV during female meiosis

Because recent work has identified heterochromatin tethers that can incorporate the AATAT repeat (Hughes *et al.* 2009), we asked whether these tethers were also present in *D. simulans*. We were able to detect them by both a phospho-specific histone antibody that can highlight threads (Hughes *et al.* 2011) and by FISH of an AATAT probe (Figure 2). However, during this experiment, we noticed that it was much more difficult to find oocytes that had their chromosome *4*s positioned far enough out on the spindle to have detectable threads, in both *D. simulans* and introgressed *sim-IV* females. Instead, while roughly similar numbers of oocytes appeared to have chromosomes out on the spindle (and therefore also roughly equal durations of time spent in prometaphase), those chromosomes were positioned much closer to the main mass of chiasmate chromosomes. To quantify this, we did preps under tightly controlled aging and dissection conditions and measured the *4-4* distances for oocytes from pure-strain *D. melanogaster*, introgressed *sim-IV* hetero- and homozygotes, and pure-strain *D. simulans* (Figure 3). To limit consideration to *4-4* distances under comparable conditions of prometaphase and congression, we excluded those oocytes where other chromosomes besides the *4* were spontaneously nonexchange, as well as oocytes that were fixed while chromosomes were in transient configurations such as having both homologs on the same side of the spindle (Hughes *et al.* 2009) or in the slippage configuration where the chiasmate autosomes are positioned end to end (Hughes *et al.* 2011).

Consistent with our initial qualitative observations, we found that the mean *4-4* distances in pure-strain (*pol/pol*) *D. melanogaster* females (11.3 μm) were nearly twice as large as in *D. simulans* (6.1 μm). Interestingly, the introgressed *sim-IV* chromosome was more intermediate when homozygous in *D. melanogaster* (*sim-IV/sim-IV*: 8.1 μm), suggesting that genetic background affects chromosome positioning. This may also contribute to the difference between the two heterozygous genotypes (*sim-IV/pol*: 8.7 μm, *sim-IV/ci*^{D}: 6.79 μm). Note that because the *4* chromosomes are normally positioned near the centromeres of the other chromosomes, the minimum *4* separation is the normal karyosome width, ~4.5 μm. Therefore the proportional separation of *4* chromosomes from the main mass is considerably larger in pure-strain *D. melanogaster*. Many of these comparisons, including all comparisons involving pure-strain *D. melanogaster*, were highly statistically significant as determined by pairwise *t*-tests (supporting information, Figure S1).

Table 2 Tests for *sim-IV* nondisjunction in multiple genetic backgrounds

Genotype	Normal progeny	*4*-only NDJ	*X*-only NDJ	*X* and *4* double NDJ	*pol*$^{+}$ *minute*[a]	*pol*$^{-}$ *minute*[a]	*X* NDJ %[b]	*4* NDJ %[b]
y w; sim-IV/sim-IV	181	0	0	0	0	—	0	0
y w; sim-IV/ci^{D}	230	0	0	0	2	—	0	0
y w; sim-IV/pol	1641	0	0	0	119	56	0	0
y w/y w nod[a]*; pol*	509	2	0	0	—	235	0	0.39
y w/y w nod[a]*; sim-IV/pol*	866	4	1	0	133	135	0.23	0.46
FM7/y w; sim-IV/pol	1405	1	5	1	189	134	0.85	0.21
FM7/y w; sim-IV/sim-IV	1127	3	7	0	314	—	1.22	0.26

Females of the indicated genotypes were crossed to *C(1;Y), v f B/O*; *C(4)RM, ci ey*^{R}*/O* males.

[a] The missing class of *minutes* cannot be produced by these crosses.

[b] The number of *X* NDJ progeny was doubled for calculation of *X* NDJ, to account for inviable classes (Zeng *et al.* 2010). Number of *X* and *4* double NDJ progeny was therefore also doubled for calculation of both *X* NDJ and *4* NDJ. In calculating percentage of *X* NDJ and *4* NDJ, the number of NDJ progeny was divided by the sum of the total progeny, not including *minutes*.

This novel observation that the *4th* chromosomes from these two closely related species have notably different behavior provides strong evidence that the amount of heterochromatin on a chromosome has a functional consequence. A speculative further interpretation is that if the repeats on a chromosome are forming threads that connect nonexchange homologs, then having a greater amount of those repeats may increase thread length and enable those homologs to move farther apart from each other before the tether pulls tight enough to prevent further movement.

Reducing AATAT content also affects positioning of D. melanogaster 4

This simple model suggests that deleting some of the *4* heterochromatin should reduce the *4-4* distance during prometaphase. Few deletions on the *D. melanogaster 4* chromosome are available, but *Df(4)M101-62f* deletes proximal gene-containing sequence and extends into the centromeric heterochromatin for an unknown distance (J. Locke, personal communication). We crossed this deletion to the same *pol* stock used above to produce *Df(4)m101-62f/pol* females. We found that the deficiency chromosome was noticeably smaller than *pol* and hybridized less strongly to the AATAT FISH probe (Figure 4), consistent with the deletion of some of the *4* heterochromatin. Then, we measured the *4-4* distances in oocytes from *Df(4)m101-62f/pol* females and found a highly significant reduction in the mean *4-4* distance (6.8 μm, Figure 3 and Figure S1). These results strongly support our conclusion that *4-4* distances are proportional to the amount of *4* heterochromatin.

Segregation of sim-IV in D. melanogaster females

To test whether *sim-IV* segregates properly in a foreign species, we assayed *sim-IV* by making it heterozygous over a *y*$^{+}$-marked *D. melanogaster* reference chromosome in *D. melanogaster* females. We also performed in parallel a control cross using a *w*$^{+}$-marked *D. melanogaster* chromosome *4* that was heterozygous over the same reference chromosome (Figure 5). Over 4400 progeny were scored in each experiment (Table 1). In the control cross the two progeny classes were not significantly different from the expected 1:1 ratio. In the experimental cross, *sim-IV* progeny were recovered at slightly below Mendelian expectations (47.8%). This deficit, however, is significantly below 50% ($P < 0.002$, binomial simulation). The experiment and control are also significantly different when compared directly in a contingency table ($P < 0.05$, chi square).

Normal disjunction of sim-IV in D. melanogaster

These differences might reflect a true segregation disadvantage of *sim-IV*, but also could result from small viability differences between *D. melanogaster* flies heterozygous for *sim-IV vs. mel-IV* that cannot be easily detected. We therefore performed a range of additional assays. First we measured NDJ within the above cross, since it can result from chromosome loss, the most plausible cause of reduced transmission. The absolute rate in *sim-IV/y*$^{+}$ females was 2.3×10^{-4}, lower than in the corresponding control and consistent with wild-type rates for pure-strain *D. melanogaster* from other published studies (see Figure 5).

We further tested the meiotic behavior of *sim-IV* by crossing to males from a standard NDJ tester stock that allows estimation of both *X* and *4* NDJ. We observed no *X* or *4* NDJ within the *sim-IV* introgression stock, either as *sim-IV/ci*^{D} heterozygotes or *sim-IV/sim-IV* homozygotes (Table 2). We also outcrossed the stock to a standard laboratory stock with the *4th* chromosome marked with *pol*, to create *sim-IV/pol* females, and again saw no *X* or *4* NDJ in this genotype. Because of these negative results, we considered the possibility that any defect in *sim-IV* may be weak. We reasoned that if this were the case, we might see NDJ if we sensitized the genetic background to increase NDJ, as has been done for assaying natural variation (Zwick *et al.* 1999). We performed two sensitizations, one by testing *sim-IV* in a background carrying a single dose of the meiotic mutant *nod*, and the other by testing *sim-IV* in females heterozygous for the *X* chromosome balancer *FM7*. Even in these sensitized backgrounds, we saw no increase in NDJ (Table 2). Furthermore, the transmission rates appear roughly equal for both *4th* chromosomes, by comparing the *pol*$^{-}$ *minute* and *pol*$^{+}$ *minute* progeny of heterozygous *sim-IV/pol* females. Therefore, the genetic evidence from a range of genetic backgrounds strongly suggests that the introgressed *sim-IV* chromosome is fully competent for normal segregation in female meiosis.

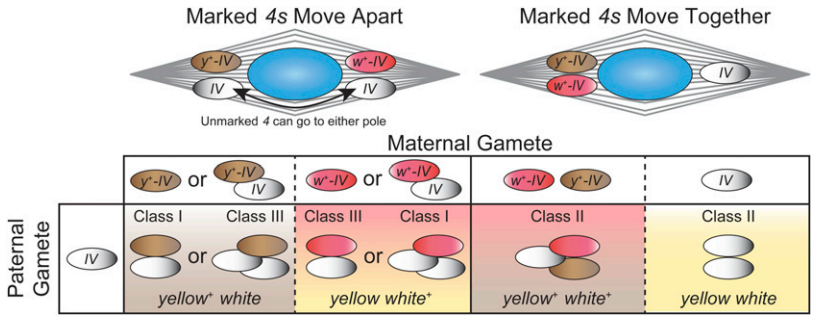

Figure 6 Expected segregation types and phenotypic classes of progeny from triplo-*4* females. The unmarked *4* being tested is indicated as "*IV*." Triploid females of chromosome *4* genotype y^+-*IV*/w^+-*IV*/*IV* were mated to *y w*/*Y* males with unmarked *4*s. Female chromosome *4*s can segregate in three possible classes to generate six different gametes. However, not all gametes can be distinguished because the tested *4* is unmarked, leading to the same phenotype from different genotypes, as indicated by background colors. When the two marked *4*s segregate to opposite poles, the unmarked chromosome will segregate to either pole. This leads to class I segregations (y^+-*IV* <=> w^+-*IV*/*IV*) and class III segregations (w^+-*IV* <=> y^+-*IV*/*IV*), which both produce progeny carrying only one of the two *4*-linked markers. Conversely, in class II segregations, the two marked *4* chromosomes move to the same pole, leading to progeny that are either wild type or mutant for both markers together. If segregation is equal, then all six classes of progeny are equally likely, leading to an expected 2: 2: 1: 1 ratio of the phenotypes y^+ *w*: *y* w^+: y^+ w^+: *y w*.

Normal sim-IV segregation in triplo-4 D. melanogaster females

Females carrying three chromosome *4*s are viable and fertile. Such females are expected to produce three types of meiotic segregation at equal frequencies (Figure 6). Sturtevant (1934, 1936) discovered, however, that in many crosses with triplo *4*s, the segregation ratios differ substantially from equal frequencies. He further determined that different chromosome *4*s from wild-type and marker strains display a characteristic "preference" for whether they tend to segregate with one of the other chromosome *4*s being tested (classes I and III in Figure 6), or instead, segregate away from the other two chromosome *4*s (class II). The genetic basis of this curious preference property remains unexplained. In our scheme, we arranged in a triplo-*4* female the unmarked chromosome to be tested against chromosome *4*s dominantly marked with either y^+ or w^+ (Figure 6). We reasoned that if *sim-IV* is perceived by *D. melanogaster* as being a foreign chromosome, then the two marked *D. melanogaster* *4*s would segregate away from each other and *sim-IV* would segregate analogous to a free duplication. This would result in a deficit of type II segregation below the random expectation of 1/3, which would manifest as a deficit of y^+ w^+ and *y w* phenotypes.

Contrary to this expectation we found that class II segregations were significantly overrepresented with *sim-IV*, but also in four of the five control crosses with *D. melanogaster* chromosome *4*s derived from different marker and wild-type stocks (Table 3). The one outlier with a significant deficit of class II segregations involved chromosome *4* from the wild-type stock BS 1. The wide range of values is consistent with results from Sturtevant (1936). This variation is not due to aberrant production or recovery of the two reciprocal classes within the three segregation types, because in most crosses the number of y^+ progeny was similar to w^+ progeny produced by class I and III segregations, and likewise for y^+ w^+ and *y w* progeny produced by class II segregation. Instead we conclude that *sim-IV* segregation falls within the normal range of variation observed for *D. melanogaster* chromosome *4*s.

Discussion

The function of heterochromatic threads in meiosis

The heterochromatic threads connecting homologous chromosomes in female meiosis are the leading candidate mechanism for how nonexchange chromosomes achieve proper coorientation (Hughes *et al.* 2009), as they can explain a variety of experimental observations, such as heterochromatic homology being sufficient to achieve segregation (Hawley *et al.* 1992). We found that *sim-IV* has shortened *4-4* distances. and is positioned more closely to the other chromosomes compared to *mel-IV*. We suggest that this correlation reflects a role of threads in chromosome positioning, but acknowledge that differential positioning might have other causes such as variation in microtubule capture or centromere strength. Regardless, we have also found that both properties correlate with differences in heterochromatin abundance, both between *mel-IV* and *sim-IV*, and between wild-type *mel-IV* and a heterochromatic deletion. Our results therefore provide evidence that the amount of heterochromatin on the *4* changes its positioning.

In addition to unresolved questions of the proximal mechanism (such as how threads are established, how they regulate coorientation, and how they are finally resolved), there is also the evolutionary question of why these chromosomes move out on the spindle at all. We suggest that because chromosome *4* is fully achiasmatic, it may be acting as an "organizing center" for threads emanating from other chromosomes. This idea is conceptually similar to a proposal by Carpenter (1991), with chromatin threads fulfilling the role previously proposed for interchromosomal microtubules. There is some circumstantial evidence for this organizational role; for example, the microtubule mass along the spindle arc between prometaphase *4* chromosomes is substantially denser than elsewhere in the spindle (Hawley and Theurkauf 1993) and in some figures, threads that appear to originate from other chromosomes can also lead toward the *4*s (Hughes *et al.* 2009). We further suggest that increased amounts of heterochromatin on *4* cause longer threads. These longer threads may more efficiently capture or associate with heterochromatic threads from facultatively achiasmate chromosomes and increase their probability of correct segregation.

Table 3 Triplo-*4* segregation tests

Source of Chr. *4* tested	No. y^+	No. w^{+a}	No. y^+ w^+	No. y w^b	% class II freq.^{c}
BS 1	725	714	285	307	29.1***
BOG 1	165	216**	151	133	42.7***
sim-IV	356	383	295	333	45.9***
VAG 1	151	167	136	171*	49.1***
Wild 5B	141	131	140	131	49.9***
y w	670	760*	741	901***	53.5***

y w; *y*$^+$*-IV* / *w*$^+$*-IV* / *4* females, where *4* represents the unmarked chromosome *4* being tested, were crossed to *y w*/*Y* males. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ in chi-squared tests.

[a] y^+ and w^+ classes were tested for deviation of a 1:1 ratio.

[b] y^+ w^+ and *y w* classes were tested for deviation of a 1:1 ratio.

[c] y^+: w^+: y^+ w^+: *y w* classes were tested for deviation from a 2:2:1:1 ratio.

If so, this role suggests parallels between the evolution of heterochromatin and other aspects of meiosis. While *D. melanogaster* has many common polymorphic chromosome inversions, *D. simulans* is monomorphic with no common inversions (Lemeunier and Aulard 1992). As inversions block crossing over, increasing the abundance of inversions will make meioses with nonexchange chromosomes more common. In *D. melanogaster*, nonexchange chromosomes move out on the spindle during prometaphase I. While the significance of this movement is not known, we speculate that it may be involved in how the oocyte achieves proper nonexchange chromosome coorientation and metaphase-arrested karyosome structure. Because nonexchange chromosomes in *D. melanogaster* are positioned between the *4*s near the spindle poles and the exchange chromosomes at the metaphase plate, having the *4*s further out would provide more space for additional nonexchange chromosomes to also move fully out onto the spindle. If this additional space is beneficial (such as reducing the time needed to complete prometaphase, or avoiding deleterious entanglements between multiple nonexchange chromosomes), then the greater amount of space on the spindle provided by the longer *4-4* tethers in *D. melanogaster* may help this species to tolerate common inversions. Note that the causal relationship in this model is unknown; it could be that longer *4-4* tethers evolved first, which allowed inversions to accumulate in the population, or alternatively, accumulating inversions favored the evolution of longer tethers to accommodate their segregation. Either way, this model predicts that *Drosophila* species with common inversions should have greater *4-4* distances than species that lack them. This would be particularly interesting to examine in species such as *D. virilis*, which has a large genome with a high satellite DNA content (Bosco *et al.* 2007), yet appears to lack inversions in natural populations (Evgen'ev *et al.* 2000). This hypothesis also may explain why dot chromosomes persist in many *Drosophila* species (Ashburner *et al.* 2005).

Heterochromatin divergence and meiotic drive

There is a resurgence of interest in heterochromatin variation, due to evidence that it affects gene expression (Lemos *et al.* 2010) and to new methods to detect and quantitate such variation (Aldrich and Maggert 2014). Strong meiotic drive is typically associated with cytologically detectable differences in heterochromatin between chromosomes (Fishman and Saunders 2008; Dawe 2009). Our results here show that a large difference in abundance of the AATAT satellite between *D. simulans* and *D. melanogaster* chromosome *4*s does not result in similarly dramatic levels of meiotic drive. We suggest that location as well as abundance influences whether satellite DNA blocks affect centromere behavior or take on neocentromere function, analogous to heterochromatin position effects that are proposed to influence whether or not circularized sex chromosomes cause mitotic defects (Ferree *et al.* 2014). Our results further suggest that strong meiotic drive is not an inevitable consequence of even extensive chromosome divergence. It remains an open question whether meiotic drivers are truly rare in nature, or instead whether higher frequency variants exist that cause lower level drive that is beyond the limit of detection in small-scale experiments. A major hurdle in resolving this question is the difficulty of reliably detecting weak meiotic drive effects, one example being the maize chromosomal knob K10L2 (Kanizay *et al.* 2013).

Faithful segregation of sim-IV

Our diplo-segregation assay did reveal a small (~2%) but statistically significant deficit in *sim-IV*-containing progeny. However this deficit is well within the range of potential viability effects. Distinguishing subtle viability effects *vs.* a meiotic segregation difference would require precise tracking and quantification of egg to adult viability for many thousands of animals. We instead pursued two additional approaches to examine *sim-IV* segregation. First we quantitated nondisjunction in a manner that includes the detection of chromosome loss events. We found no excess in NDJ for *sim-IV*, most strikingly even when sensitizing the genetic background using either a *nod* mutation or an achiasmate *X* chromosome balancer.

Segregation of sim-IV in triplo-4 females

Our second approach took advantage of the very high levels of nonrandom disjunction that are often seen in triplo-*4* females. We constructed *D. melanogaster* females containing *sim-IV* as the tester chromosome and two marked *D. melanogaster 4*s, as

well as five control lines with different tester *D. melanogaster 4s*. We expected that if *sim-IV* is "perceived" as being foreign or distinct from *D. melanogaster 4*s, then the two *D. melanogaster 4s* would preferentially segregate away from each other, resulting in an excess of class I and III segregations and a deficit of class II (Table 3). Instead we saw the opposite pattern, with 45.9% class II segregations compared to the random expectation of 33.3%.

It is instructive to compare this result to cases where chromosome *4* derivatives or aberrations have been introduced into diplo-*4* backgrounds, even if the use of different reference *4s* between studies precludes precise quantitative comparisons. Table 3 in Hawley *et al.* (1992) examined the effects of a series of *Dp(1;4)* chromosomes containing varying amounts of chromosome *4* heterochromatin on segregation of two marked chromosome *4*s. NDJ of these two *4s* is analogous to class II segregation in Figure 6. NDJ ranged from ~12 to 33% and showed a positive correlation with abundance of chromosome *4* heterochromatin. Interestingly, a deletion derivative, *Dp(1;4)M5D*, that appears to remove some chromosome *4* heterochromatin induced very low NDJ. Similarly, Bauerly *et al.* (2014) recently discovered *D. melanogaster* strains containing *B* chromosomes that are predominantly composed of AATAT satellite and may be derived from chromosome *4s*. These *B* chromosomes induced 27.1% chromosome *4* NDJ. These results make all the more striking the fact that *sim-IV* induces a very high frequency of class II segregations despite having reduced AATAT content.

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Normal Segregation of a Foreign-Species Chromosome During *Drosophila* Female Meiosis Despite Extensive Heterochromatin Divergence

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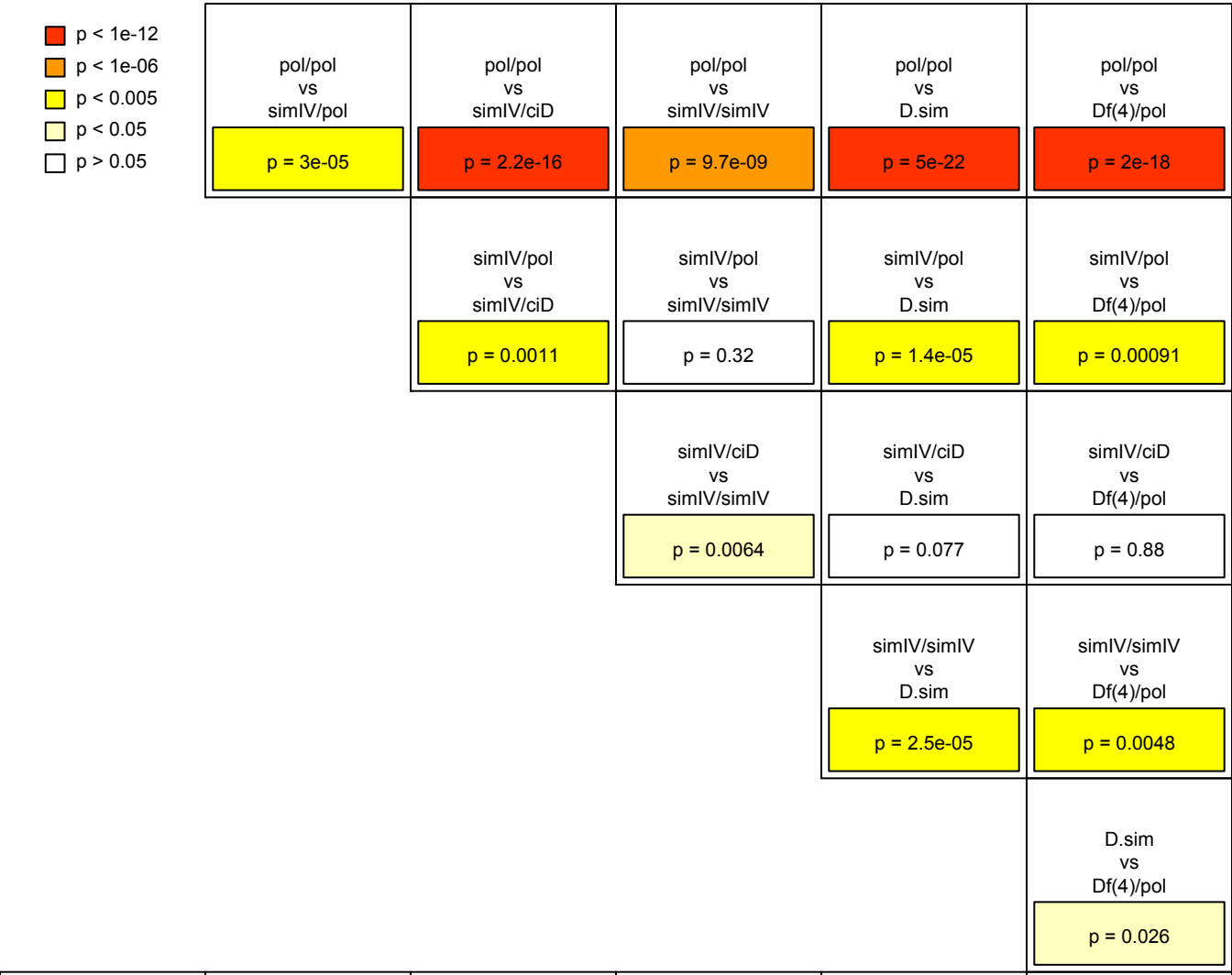
p < 1e-12
p < 1e-06
p < 0.005
p < 0.05
p > 0.05
pol/pol vs simIV/pol
p = 3e-05
pol/pol vs simIV/ciD
p = 2.2e-16
pol/pol vs simIV/simIV
p = 9.7e-09
pol/pol vs D.sim
p = 5e-22
pol/pol vs Df(4)/pol
p = 2e-18
simIV/pol vs simIV/ciD
p = 0.0011
simIV/pol vs simIV/simIV
p = 0.32
simIV/pol vs D.sim
p = 1.4e-05
simIV/pol vs Df(4)/pol
p = 0.00091
simIV/ciD vs simIV/simIV
p = 0.0064
simIV/ciD vs D.sim
p = 0.077
simIV/ciD vs Df(4)/pol
p = 0.88
simIV/simIV vs D.sim
p = 2.5e-05
simIV/simIV vs Df(4)/pol
p = 0.0048
D.sim vs Df(4)/pol
p = 0.026
pol/pol
Total = 180
% out = 48.3
simIV/pol
Total = 69
% out = 56.5
simIV/ciD
Total = 88
% out = 39.8
simIV/simIV
Total = 178
% out = 29.8
D.sim
Total = 99
% out = 44.4
Df(4)/pol
Total = 210
% out = 22.4

Figure S1 Significance of *4-4* distance measurements. The *4-4* distances for each genotype in Fig. 3A were used to calculate all possible pairwise *t*-tests, with genotypes in the same order as in Fig. 3A. The *p* values for each test are listed and color coded according to the key. The bottom row shows the total number of oocytes examined, along with the percentage of those oocytes with 1 or more chromosomes out on the spindle (which indicates that the oocyte is in prometaphase) for each genotype. Note that the number of oocytes out ("% out" times Total) is greater than the N values listed in Fig. 3A, as oocytes with nonexchange chromosomes in addition to *IV* or transient configurations such as slippage or with both homologs on the same spindle arm were excluded from the *4-4* measurements, but were counted here.